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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.

Cab-001

Total Pages

20

First Named Inventor or Application Identifier

Portugal, Frank, H.

Express Mail Label No.

EI071343975US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages]
(preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☐ Drawing(s) (35 USC 113) [Total Sheets]
4. Oath or Declaration [Total Pages]
 - a. ☒ Newly executed (original or copy)
 - b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

ADDRESS TO:

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6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☒ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☐ Return Receipt Postcard (MPEP 503)
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Statement(s) Status still proper and desired
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PATENT

Attorney Docket

Cab-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

SPECIFICATION

accompanying

Application for Grant of U.S. Letters Patent

INVENTOR: Frank H. Portugal

TITLE: METHOD FOR IDENTIFYING SPECIES OF SHIGELLA
AND E. COLI USING OPERON SEQUENCE ANALYSIS

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Date of Deposit February 20, 1998

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Sir:

Re: New U.S. Patent Application for METHODS FOR IDENTIFYING SPECIES
OF *SHIGELLA* AND *E. COLI* USING OPERON SEQUENCE ANALYSIS
Inventor: Frank H. PORTUGAL
Filing Date: February 20, 1998
Atty Docket No.: Cab-001

Please accord the enclosed new U.S. Patent application a serial number and filing date.

Enclosed are:

- 1) Utility Patent Application Transmittal form;
- 2) Specification, including 20 total pages and 7 total claims;
- 3) Executed Declaration;
- 4) Executed Small Entity Statement;
- 5) Check for \$ 395.00, the basic filing fee for a small entity; and
- 6) a postage-paid return address postcard.

Please address all correspondence to:

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Please notify the applicant at the address above of any missing parts of the this application.

Respectfully Submitted,


David J. Kulik, Reg. No. 36,576

ABSTRACT

A method for comparing the variable reactivity of multiple, differentially mutated copies of 16S subsequences found in a number of ribosomal RNA operons of a single bacterial cell is described. The application of this method for distinguishing between closely related organisms, such as the genera *Escherichia* and *Shigella*, and between species of *Shigella* including *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* using nucleic acid probes is also presented.

NUCLEIC ACID PROBES FOR THE DETECTION OF SHIGELLA

FIELD OF THE INVENTION

This invention relates to a method for distinguishing among bacteria within the same taxonomic group based on the reactivity of specific 16S subsequences found within the ribosomal operons of the organisms, using probes during hybridization under conditions of increasing severity (stringency). Hybridization is the process whereby two strands of nucleic acid can interact and, if sufficiently matched in sequence, form a double-stranded structure. By the term probe is meant a marked, single-stranded nucleic acid sequence that is complementary to the nucleic acid sequences to be detected (target sequences). The use of this method of operon analysis for distinguishing the genera *Escherichia* from *Shigella* and for distinguishing among species of *Shigella* is demonstrated together with nucleic acid probes needed for conducting the analyses.

BACKGROUND OF THE INVENTION

The terms "*Escherichia coli*," "*Shigella boydii*," "*Shigella dysenteriae*," "*Shigella flexneri*," and "*Shigella sonnei*" refer to the bacteria classified as such in Bergey's Manual of Determinative

Bacteriology, 8th Edition, R.D. Buchanan and N.E. Gibbons, Eds., William & Wilkins, 1974, pp. 290-339. Unless specified otherwise the term *Shigella* will refer collectively to the four species mentioned above.

Detection of *Shigella* is important for medical diagnosis, public health surveillance, food safety, and other applications. Cases of *Shigella*, which must be identified by species, are required to be reported to the Centers for Disease Control and Prevention, which tracks the incidence and prevalence of *Shigella* in every state in the United States and in the District of Columbia. Current methods of detection are neither simple, straightforward, nor absolute (J.J. Farmer, III and M. T. Kelly, Enterobacteriaceae, in A. Balows, Ed, Fifth Edition, Manual of Clinical Microbiology, Washington, D.C., American Society for Microbiology, 1991.)

Suspected colonies usually are grown on both MacConkey agar and xylose-lysine-deoxycholate agar. Colonies of *Shigella* missed on one medium may show up on the other. Many laboratories also use Hektoen enteric agar. Enrichment of less than optimum cultures may require GN broth. Selenite broth may be useful for isolating *S. sonnei*. Suspected colonies of *Shigella* require confirmation by culture on other types of growth media such as triple sugar iron or Kligler iron agar slants. Colonies that show an alkaline/acid reaction with no H₂S or gas then must be screened further by serological analysis with antisera in order to identify each of the species of *Shigella*.

Even with these procedures, differentiating strains of *Shigella* from *Escherichia coli* has proved to be one of the most difficult problems for a clinical microbiology laboratory. Recommended guidelines are complicated by exceptions related to one or more *Shigella* species. The difficulties inherent in distinguishing these organisms often forces investigators to depend merely on the fact that two

Shigella species (*S. boydii* and *S. flexneri*) are not as prevalent in the United States, although a significant number of cases do occur, as are the other two species in order to help solidify their diagnoses. Still, the guidelines conclude with the realization that no definitive rules on the identification of *Shigella* isolates can be made and complete biochemical and serological typing must be done in each instance.

It is yet another aspect of the invention to avoid the disadvantages associated with the traditional culturing and serological identification techniques and to employ nucleic acid probes to distinguish *Escherichia coli* from *Shigella* and to identify each of its four associated species.

Efforts to circumvent the difficult, expensive, and time-consuming procedures with a simple yet rapid molecular procedure for differentiating the genus *Shigella* from the genus *Escherichia* and for separately identifying each of the four individual species of *Shigella* has also proved difficult. This has been attributed, in particular, to the very close relatedness of *E. coli* and all four *Shigella* species by DNA-DNA hybridization (J.J. Farmer, III and M. T. Kelly, Enterobacteriaceae, in A. Balows, Ed, Fifth Edition, Manual of Clinical Microbiology, Washington, D.C., American Society for Microbiology, 1991.)

Kyriaki Parados and Janice McCarty (U.S. Patent Number 5,648,481) have identified a set of nucleic acid probes for detection of the genus *Shigella* and/or *E. coli* (EIBC) based on specific chromosomal sequences and fragments of *Shigella*. These probes can neither distinguish between *E. coli* and *Shigella* nor can they separately distinguish between one species of *Shigella* and another. In addition, the relatively large probes (approximately 40 nucleotides each) require hybridization overnight followed by exposure for 15 hours to x-ray film to produce autoradiographs.

Alessio Fasano, Myron M. Levine, James P. Nataro, and Fernando Noriega (U.S. Patent Number 5,589,380) targeted the enterotoxins of *Shigella flexneri* 2a and produced antibodies to the same, which might be useful primarily for the identification of *S. flexneri*. Kyriaki Parodos, Hsien-Yeh Hsu, Daid Sobell, Janice M. McCarty, and David J. Lane (U.S. Patent Number 5,084,565) devised probes capable of hybridizing to rRNA of both *E. coli* and *Shigella* species but unable to discriminate among them. Phillippe Sansonetti, Catherine Boileau and Hélène D'Hauteville (U.S. Patent Number 4,992,364) targeted the 140 MDalton virulence plasmid of *S. flexneri*. Their probes are relatively large, ranging in size from about 11.5 kbases to 27 kbases, and identify only combined strains of *Shigella* and *E. coli* carrying the virulence plasmid. Long-term hybridization (overnight) is followed by 6 hours of exposure to produce autoradiographs.

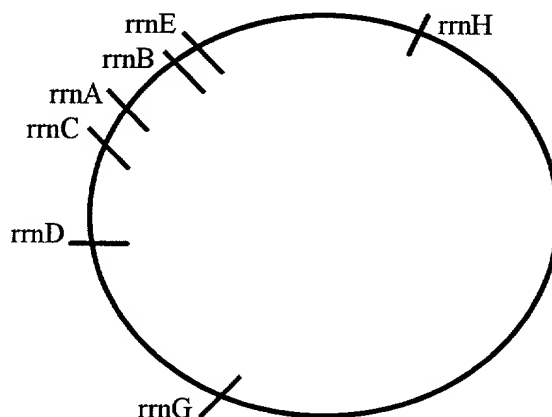
A subsequence of ribosomal RNA (rRNA) or its gene presents a potential target for separate identification of *E. coli* and each of the four species of *Shigella* through hybridization with appropriate DNA or RNA probes. Portions of rRNA have been found not to be conserved among diverse bacterial species, making them potential hybridization targets for distinguishing between one taxonomic group and another. David E. Kohne (U.S. Patent Number 5,601,984) discusses such a method for detecting and quantitating organisms. But Kohne does not provide the teaching necessary to make *Shigella* species-specific probes.

Furthermore, Kohne does not teach how to distinguish among very closely related organisms using probes where a subsequence of a rRNA subunit or rRNA subunit gene is *not* specific to the taxonomic group (qualitative difference) but rather occurs as multiple but slightly differentiated copies in different proportions among multiple operons for the RNA genes (quantitative difference). An operon is defined as a group of contiguous genes that are coordinately regulated by controlling

elements. Nor does Kohne teach the use of probes of sequence specific neither to genus nor species or other taxonomic grouping.

The *E. coli* chromosome is circular and contains seven operons for rRNA (Figure 1). A typical rRNA (rrn) operon contains two promoters and genes for 16S, 23S, and 5S rRNA and a single 4S tRNA gene (Figure 2). When analyzed, the 16S genes of the different *E. coli* rrn operons have been found to have regions where the sequences have been altered through mutations (Table 1). In some operons the mutations are the same in one particular region and in other operons they are different. Other organisms such as *Shigella* may either have a different number of operons, different types of operons, a different proportion of a particular mutation in one or more of its operons, one or more mutations in its operons distinct from *E. coli*, or all of these possibilities.

Figure 1
Organization of the Ribosomal RNA Operons on the *E. coli* Chromosome



Each line marks the relative position of one of the seven rrn operons found on the *E. coli* chromosome.

Figure 2
Internal Organization of a Ribosomal RNA Operon

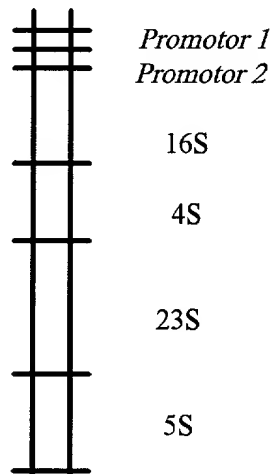


Table 1
Subsequence Variation in the Ribosomal RNA Operons of *E. coli*

Operon or Strain	Nucleotide Position	Sequence Variant			
ECORRD	71-100	AACAGGAAGA	AGCTTGCTCT	TT	GCTGACGA
rrnG	71-100	-----AC	-----G-	---/C\-----	
rrnE	71-100	-----	-----/T\--	--	-----
rrnC	71-100	-----AC	-----	---/C\-----	
rrnB	71-100	-----	-----/T\--	--	-----
ATCC 25922	71-100	-----CG	-----/G\--	--	-----
ATCC 11775T	71-100	-----C	-----/G\--	--	-----

The number of possible potential combinations suggests that hybridization of rrn operon subunit probes specific for 16S does not detect subsequences specific to a taxonomic unit but instead detects a variable number of a targeted mutation within the operons, the number and variability of which may differ from one closely related organism to another. Thus, the rrn operon subsequences for 16S not only exhibit phylogenetic variability between organisms but variability even within a given genus and species of a single organism.

Sequencing of 16S genes in rrn operons does not necessarily clarify the variability seen. The entire process of preparing a plasmid vector containing ligated genetic material from rrn operon 16S genes

in order to conduct the sequencing studies fails generally to select either for a specific operon or for a single bacterial cell containing a vector carrying a single rrn operon. DNA representing the rrn operon 16S subsequences, for example, prepared from *Shigella* is amplified in the polymerase chain reaction (PCR) using universal primers that are unlikely to discriminate between subsequences from one operon and another. Competent cells carrying the ligated vector are then selected for large-scale cloning. A colony of cells, rather than a single cell or cell clone, is chosen.

In all cases of selection for the sequencing process, heterogenous rrn operon material rather than homogenous rrn operon nucleic acid may be obtained. The resulting genetic sequences may either then represent a broad consensus sequence from all the possible rrn operon subsequences or a skewed sequence representing some but not all of the rrn operons and their different subsequences. This makes it very difficult to predict from the rrn sequences which probe subsequences are most likely to differentiate between one taxonomic group and another.

Hybridization refers to the process whereby sequence-specific, base-paired duplexes from any combination of nucleic acid fragments are formed. A useful measure of the stability of a DNA duplex or an RNA-DNA hybrid is the melting temperature or T_m , which refers to the temperature at which the strands are half dissociated or denatured. Complexity refers to the total length of different sequences present in a sample of nucleic acid. Reassociation is the process of joining together by typical base pairing the two fully separated complementary sequences. Complementary refers to rules of base pairing enunciated by Erwin Chargaff whereby an adenine base pairs with its thymine complement and a guanine base pairs with its cytosine complement.

Hybridization reactions are usually done in a buffered, aqueous medium that may contain other additives. Additives may include detergent, salts, polymers, and blocking agents. The stringency of the hybridization medium may be controlled by temperature, salt concentration, probe concentration, probe length, time, and other factors. The rate of reassociation of two simple DNA strands with complementary sequences and no significant sequence repetition is easily described by practical kinetic equations such as (1) and (2) given below.

$$H = (1 + kC_0t)^{-1} \quad (1)$$

where H = fraction of DNA not bound to hydroxyapatite, k = observed rate constant, C_0 = original concentration of nucleotides, and t = time in seconds. Hydroxyapatite is used to distinguish single-stranded DNA from double-stranded DNA.

$$S = (1 + kC_0t)^{-0.44} \quad (2)$$

where S = fraction of nucleotides remaining unpaired based on the use of nuclease S1 to differentiate single and double-stranded DNA.

The kinetics describing the rate of reassociation for complex genomes with sequence repetition is more difficult, particularly if bound to a filter or other surface such as an optic fiber. Although bacteria have relatively simple genomes compared to higher organisms, the repetition and multiplicity of the *rnm* operons in bacteria make their hybridization reactivity difficult to predict. For example, as stringency is increased, sequences that are not perfectly complementary should become less stable. The extent of hybridization of a probe to a given bacterial genome, therefore, might be

expected to decrease with increasing temperature if there were some mismatching of bases or, at best, stay the same if there was perfect complementarity.

Stringency washes are usually performed at 3-5° C below the T_m of the perfectly matched probe when differentiation from mismatched sequences is required. The Wallace rule (R.B. Wallace and C.G. Miyada, *Methods in Enzymology* 152:438, 1990) can be used as follows to calculate the T_m for a probe in order to set the temperature conditions necessary to avoid mismatched sequences:

$$T_m = (4 \times \text{number of G + C bases}) + (2 \times \text{number of A + T bases}) \quad (3)$$

However, when the T_m is calculated in this manner for each of the probes of the present invention, it is found to be more than 5° below the actual T_m as measured in 1 M NaCl. This discrepancy further complicates a determination of stringency for *rrn* operon analysis. The method of the present invention provides conditions for hybridization of the probes that exceeds the calculated T_m and ensures that stringent conditions are being employed.

Previous studies have shown that a bell-shaped curve describes the relationship between the rate of hybridization and the temperature of incubation for formation of well-matched hybrids (Margaret L.M. Anderson and Bryan D. Young, Quantitative Filter Hybridization, in Roy J. Britten and Eric H. Davidson, *Nucleic Acid Hybridization*, Academic Press, 1985). The curves show a low relative rate of reassociation of perfectly matched sequences at either end (-50° C below the T_m and at the T_m) of the bell curve and a maximal rate of reassociation at -20° C below the T_m . The rate of reassociation for mismatched sequences falls to zero at -20° C below the T_m .

Such studies do not teach the hybridization reactivity seen when the probes of the present invention directed toward *rrn* operon 16S subsequences are hybridized at temperatures above their calculated T_m . The studies do not generally predict a loss of hybridization reactivity at temperature a few degrees below the measured T_m followed by a reappearance of hybridization reactivity as the temperature approaches the measured T_m of the probe. This phenomenon is seen, however, for some but not all of the probes used to test *E. coli* and the *Shigella* species reported herein. The reactivity of these particular probes with bacterial genomes have neither been previously disclosed or discussed.

The present invention also provides a method for organizing the complexity of probe reactivity with *E. coli* and *Shigella* organisms into a hierarchical flow diagram for identifying one or more of these closely related organisms when present in a sample.

DISCLOSURE OF THE INVENTION

The present invention provides a method and means for detecting, identifying, and quantifying *Shigella* in biological and other samples and more specifically for sensitively detecting and quantifying such organisms. For example, the 16S nucleotide subsequences of the *rrn* operons have been analyzed in order to develop probes for variable reactivity with the operons of different species of *Shigella* and *E. coli*.

Hybridization is conducted by dot blot on nylon filters, but other methods such as solution hybridization and covalent attachment to a solid surface such as an optical fiber for sandwich hybridization are possible as are variations in the composition of the prehybridization, hybridization,

SOME 16S sequences are discussed in
U.S. Provisional patent application
Serial no. 60/038,117, filed February 20
1997, specifically incorporated herein

by
Reference

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and wash buffers and the methods for detecting when hybridization has occurred such as the use of chemiluminescence, bioluminescence, fluorophores, and others. The present embodiments can also be employed using a near-infrared optical fiber evanescent field-excited fluorosensor (Saeed Pilevar et al., Serial No. 08/866.080, filed May 30, 1997).

specifically incorporated herein by reference.

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In the preferred embodiment, a method for discriminating among members of a taxonomic group by hybridization analysis of operon subsequences has been determined. Either RNA or DNA from *E. coli* and *Shigella* can serve as the target for these probes. A sample is tested for rrm operon 16S subsequence reactivity by hybridization to each probe under various conditions to ensure increasing levels of stringency. The operon subsequence reactivity is tested by using each oligonucleotide probe under controlled stringency conditions at two or more wash temperatures relative to the probe's calculated or experimentally determined T_m . The hybridization reactions are then assayed to determine the relative level of reactivity of the combined operon 16S subsequences that may be present.

Four probes for distinguishing between the genera *Escherichia* and *Shigella* and their species are the subject of the present invention. The probes are identified as follows:

- SEQ ID NO 1
- Q 1. ~~Seq. ID. Number 1~~ has the sequence CAG CTT GCT CTT CGC TGA CG.
- SEQ ID NO 2
- Q 2. ~~Seq. ID. Number 2~~ has the sequence AAA GCA GCT TGC TCT TTG CT.
- SEQ ID NO 3
- Q 3. ~~Seq. ID. Number 3~~ has the sequence CGA CGC AAC GCG AAG AAC TT.
- SEQ ID NO 4
- Q 4. ~~Seq. ID. Number 4~~ has the sequence GAA GCT TGC TTC TTT GCT GAC.

The following *Shigella* species were obtained from the American Type Culture Collection for the study: *S. boydii* (8700), *S. dysenteriae* (13313), *S. flexneri* (29903), and *S. sonnei* (29930).

Escherichia coli O157:NM (G5066) was obtained from the Centers for Disease Control and Prevention.

EXAMPLE 1

Production of a Probe That Will Hybridize and Distinguish *E. coli* and *S. sonnei* from Other Species of Shigella

A probe sequence (SEQ ID NO:1) was identified for the 71-100 nucleotide position of the *rrn* operon 16S subsequence of *S. boydii*. This regional sequence has not been reported for any of the corresponding *rrn* operon 16S subsequences of *E. coli*, for the *rrn* operon subsequence of another strain of *S. boydii*, or for the corresponding *rrn* operon subsequences for other species of Shigella. Without hybridization analysis of the *rrn* operons in *E. coli* and Shigella, it would be difficult to predict the extent to which, if any, this probe would react with these organisms. The measured T_m for the probe in 1 M NaCl is 72° C whereas the calculated T_m is 64° C.

Accordingly, DNA samples from each species of Shigella and from *E. coli* were prepared by standard phenol extraction (F. Ausubel et. al., Eds. *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989). DNA was denatured by heating on 0.2 N NaOH at 37° C for 10 minutes. Blots were prepared by spotting solutions of each DNA preparation (40 µg/blot) on Nytran filters (Schleicher & Schuell). DNA was crosslinked to the filter in a Stratalinker (Stratagene) by applying 120,000 mJoules of ultraviolet light to the surface. Filters were prehybridized with buffer consisting of 6x SSC, 3% blocking powder (Boehringer-Mannheim), and 0.5% Tween 20 heated to 60° C. For preparing the prehybridization solution, the components were added and the solution was then heated at 70° C for 20 minutes before being cooled to room temperature. 5'-Labeled biotin

probes were added directly to the prehybridization buffer, and samples were incubated for either 2 hours or overnight.

After hybridization was complete, filters were washed in 6x SSC twice for 5 minutes each time at room temperature and then once in 6x SSC for 10 minutes at the wash temperature indicated. Filters were next transferred to a detection blocking buffer that consisted of 6x SSC, 0.35% blocking powder, and 0.1% Tween 20. The filters were incubated in this buffer at room temperature for 20-30 minutes with gentle rocking.

A conjugate of streptavidin and horseradish peroxidase (KPL Labs) at a 1:500 dilution (0.1 mg/ml, 40 µl total) was added directly to the detection blocking buffer, and the filters were incubated at room temperature for an additional 20 minutes without shaking. Filters were then transferred to a clean container and washed 3 times with 1x membrane wash buffer (KPL Labs). Each wash was carried out at room temperature for 5 minutes with gentle shaking.

Just prior to use, equal volumes of the HRP detection reagents (KPL Labs) (luminol and H₂O₂) were mixed together, and then added to the filters in a clean container. After a 60-second incubation, the filters were wrapped in Saran Wrap, and exposed to Hyperfilm (Amersham) x-ray film. Exposures ranging from 10 minutes to overnight were made as needed.

Table 2—
Seq. ID: Number 1

Organism	Wash Temperature (C)		
	62°	66°	72°
<i>Shigella boydii</i>	+	+	-
<i>Shigella dysenteriae</i>	+	+	-
<i>Shigella flexneri</i>	+	+	-
<i>Shigella sonnei</i>	+	+	+
<i>Escherichia coli</i>	+	+	+

The results suggest that *E. coli* and the four species of *Shigella* have operons capable of reacting with this probe under high stringency, but that only *S. sonnei* and *E. coli* operon subsequences remain reactive at the observed T_m . Although based on sequence differences seen for the *S. boydii* operon analysis, this probe is useful for the identification of *S. sonnei* and/or *E. coli* operon subsequences when used at 72° C for DNA-DNA hybridization. The reactivity of this probe at 72° C indicates that the probe is not specific for a particular taxonomic group since it recognizes both *S. sonnei* and *E. coli* operon subsequences but fails to recognize subsequences for other members of the genus to which *S. sonnei* belongs. Although derived from *S. boydii* sequences, the probe at 72° fails to recognize *S. boydii*.

EXAMPLE 2

Production of a Probe That Will Hybridize and Distinguish *Shigella sonnei* and *E. coli* from Other Species of *Shigella* or *S. dysenteriae* from *E. coli* and Other Species of *Shigella*

a. ^{SEQ ID NO 2}
Probe ~~Seq. ID: Number 2~~ is based on a sequence determined from *S. dysenteriae*. This probe has a calculated T_m of 58° C and a measured T_m in 1 M NaCl of 66° C.

Table 3
Seq. ID: Number 2

Organism	Wash Temperature (C)		
	56°	66°	62°
<i>Shigella boydii</i>	-	-	-
<i>Shigella dysenteriae</i>	+	-	+
<i>Shigella flexneri</i>	+	-	-
<i>Shigella sonnei</i>	+	+	-
<i>Escherichia coli</i>	+	+	-

This probe also fails to distinguish *rrn* operon subsequences of either genus or species. At 56° C it reacts with *E. coli* and *S. dysenteriae*, *S. flexneri*, and *S. sonnei* but not with *S. boydii*. At 66° C it

still reacts with two different species, *S. sonnei* and *E. coli*, but not with other *Shigella* species, thereby failing to distinguish a specific taxonomic group. Under the most stringent hybridization conditions, this probe fails to react with *S. dysenteriae*, the organism from which the probe sequence was derived.

EXAMPLE 3

Production of a Probe That Will Hybridize and Distinguish *E. coli* from *Shigella*

- SEQ ID NO 3
 C The probe based on Seq. ID: Number 3 is derived from a *S. sonnei* sequence, with a calculated T_m of 62° C and a measured T_m in 1 M NaCl of 70° C.

Table 4
 Seq. ID: Number 3

Organism	Wash Temperature (C)	
	66°	70°
<i>Shigella boydii</i>	-	-
<i>Shigella dysenteriae</i>	+	-
<i>Shigella flexneri</i>	+	-
<i>Shigella sonnei</i>	+	-
<i>Escherichia coli</i>	+	+

This probe should show homology with the *S. sonnei* operon 16S subsequence but not with any of the corresponding *E. coli* operon 16S subsequences. Nevertheless, the probe crosses genus and species categories at 66° and hybridizes at 70° with *E. coli* operon 16S subsequences but not with *S. sonnei* operon 16S subsequences from which the probe was derived.

EXAMPLE 4

Production of a Probe That Will Hybridize and Distinguish *E. coli* from *Shigella*

- SEQ ID NO 4
A The probe labeled ~~Seq. ID. Number 4~~ is specific to some but not all *rrn* operon 16S subsequences ascribed to this region of *E. coli*. However, since it is unlikely that all *rrn* operon 16S subsequences for *Shigella* in this region have been sequenced for the reasons noted previously, it cannot be stated with certainty that this subsequence is specific only to *E. coli*. The T_m for this probe when calculated is 62° C and when measured in 1 M NaCl is 69° C. This probe reacts solely with *E. coli* at 69° C.

Table 5

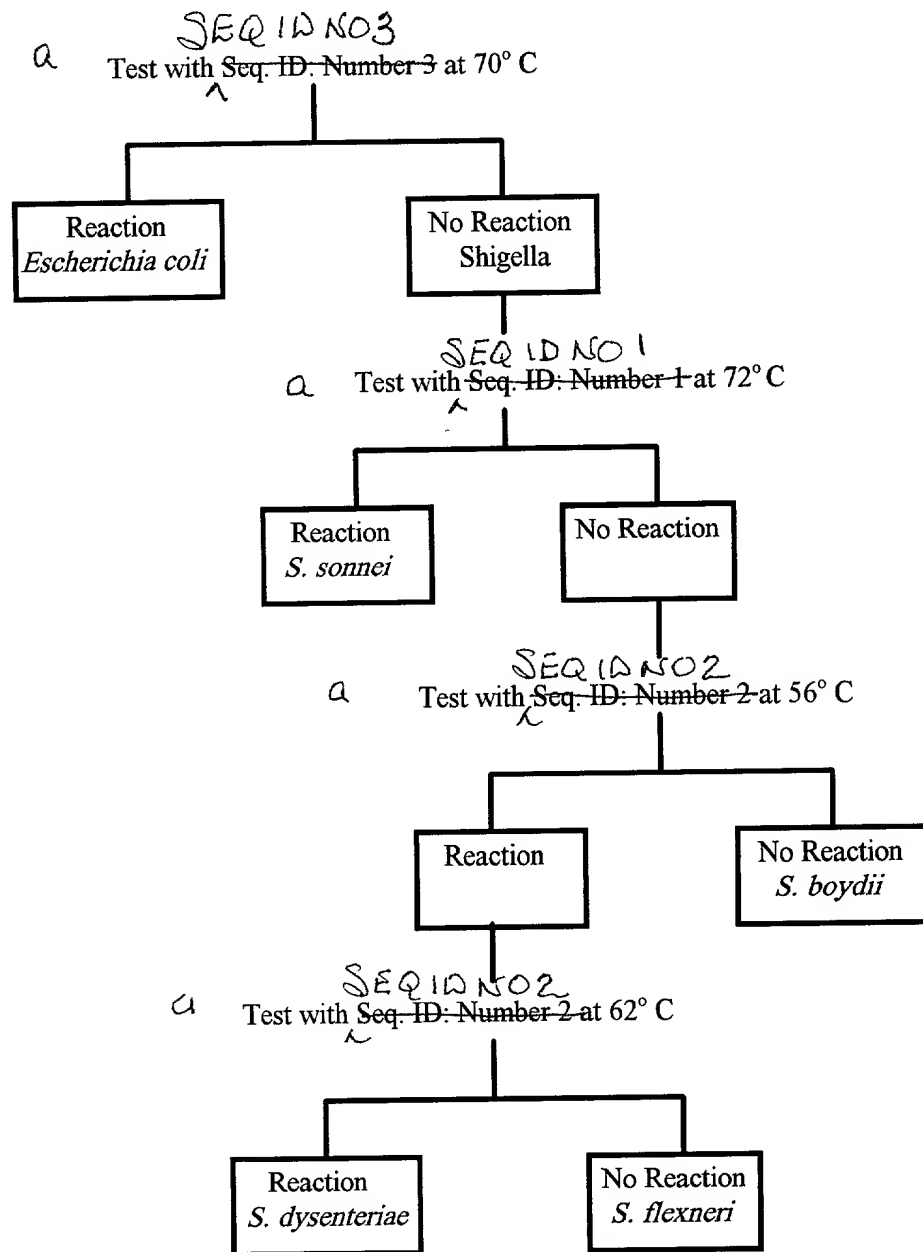
shows the specificity of the probes determined by testing each one at one or more wash temperatures with DNA prepared from each of six other species of enteric bacteria closely related to *E. coli* and *Shigella*. Samples of each bacterium were obtained from the American Type Culture Collection. The following strains were used: *Citrobacter freundii* (8090), *Klebsiella pneumoniae* (13883), *Proteus vulgaris* (13315), *Salmonella choleraesuis* (14028), *Serratia marescens* (13880), and *Yersinia enterocolitica* (9610). The results indicate that the probes do not cross-react with operon 16S subsequence variants found in any of the other organisms tested.

Table 5
Specificity of Operon-Based 16S Subsequence Probes

Probe	Temperature	Organism	Reactivity
SEQ ID NO 1 Seq. ID: Number 1	72°	<i>Citrobacter freundii</i>	-
		<i>Klebsiella pneumoniae</i>	-
		<i>Proteus vulgaris</i>	-
		<i>Salmonella choleraesuis</i>	-
		<i>Serratia marescens</i>	-
		<i>Yersinia enterocolitica</i>	-
SEQ ID NO 2 Seq. ID: Number 2	56°	<i>Citrobacter freundii</i>	-
		<i>Klebsiella pneumoniae</i>	-
		<i>Proteus vulgaris</i>	-
		<i>Salmonella choleraesuis</i>	-
		<i>Serratia marescens</i>	-
		<i>Yersinia enterocolitica</i>	-
SEQ ID NO 2 Seq. ID: Number 2	62°	<i>Citrobacter freundii</i>	-
		<i>Klebsiella pneumoniae</i>	-
		<i>Proteus vulgaris</i>	-
		<i>Salmonella choleraesuis</i>	-
		<i>Serratia marescens</i>	-
		<i>Yersinia enterocolitica</i>	-
SEQ ID NO 3 Seq. ID: Number 3	66°	<i>Citrobacter freundii</i>	-
		<i>Klebsiella pneumoniae</i>	-
		<i>Proteus vulgaris</i>	-
		<i>Salmonella choleraesuis</i>	-
		<i>Serratia marescens</i>	-
		<i>Yersinia enterocolitica</i>	-
SEQ ID NO 4 Seq. ID: Number 4	69°	<i>Citrobacter freundii</i>	-
		<i>Klebsiella pneumoniae</i>	-
		<i>Proteus vulgaris</i>	-
		<i>Salmonella choleraesuis</i>	-
		<i>Serratia marescens</i>	-
		<i>Yersinia enterocolitica</i>	-

HYBRIDIZATION STRATEGY TO SEPARATE *E. COLI* FROM SHIGELLA AND TO SEPARATELY IDENTIFY SHIGELLA SPECIES

The following strategy shown as a flow diagram below has been used to first separately determine whether a sample is *E. coli* or Shigella and then, if Shigella, to determine which of four species it is.



What is claimed is:

- Sub 137
1. A method for discriminating among members of one or more taxonomic groups by hybridization analysis of operon subsequences either as DNA or as the RNA product(s) comprising the steps of:
 - a. determining operon subsequence reactivity by testing samples with each oligonucleotide probe under controlled stringency conditions at two or more temperatures relative to the probe's calculated or experimentally determined T_m or by other changes,
 - b. contacting individually one or more samples that may contain operon subsequences with one or more oligonucleotide probes,
 - c. incubating the probes and samples at various temperatures and other conditions such that increasing degrees of stringency are obtained,
 - d. assaying for hybridization of the probes to the samples in order to determine the relative level of reactivity of the combination of operon subsequences present in each sample.
 2. The method of claim 1 for discriminating among the genera *Shigella* and *Escherichia* and their species wherein probe Seq. ID: Number 1 has the sequence CAG CTT GCT CTT CGC TGA CG. 2/20/98
F10
and is used
 3. The method of claim 1 for discriminating among the genera *Shigella* and *Escherichia* and their species wherein probe Seq. ID: Number 2 has the sequence AAA GCA GCT TGC TCT TTG CT. 2/20/98
F10
and is used
 4. The method of claim 1 for discriminating among the genera *Shigella* and *Escherichia* and their species wherein probe Seq. ID: Number 3 has the sequence CGA CGC AAC GCG AAG AAC TT. 2/20/98
F10
and is used
 5. The method of claim 1 for discriminating among the genera *Shigella* and *Escherichia* wherein probe Seq. ID: Number 4 has the sequence GAA GCT TGC TTC TTT GCT GAC. 2/20/98
F10
and is used
 6. RNA sequences for probe Seq. ID: Numbers 1, 2, 3, or 4 wherein U substitutes for T. 2/20/98
F10
and is used
 7. Variants through addition, subtraction, and/or modification of bases of probe Seq. ID: Number 1, 2, 3, or 4. 2/20/98
F10
and is used

A method for comparing the variable reactivity of multiple, differentially mutated copies of 16S subsequences found in a number of ribosomal RNA operons of a single bacterial cell is described. The application of this method for distinguishing between closely related organisms, such as the genera *Escherichia* and *Shigella*, and between species of *Shigella* including *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* using nucleic acid probes is also presented.

DECLARATION AND POWER OF ATTORNEY

As the below named inventor, I hereby declare that: My residence, post office address, and citizenship are as stated below next to my name. I am the sole inventor of the subject matter claimed for which a patent is sought on the invention entitled:

METHODS FOR IDENTIFYING SPECIES OF *SHIGELLA* AND *E. COLI*
USING OPERON SEQUENCE ANALYSIS

the specification of which is attached. I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information material to the examination of this application in accordance with Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Country (if PCT indicate PCT)	Application Number	Date of Filing (day, month, year)	Priority Claimed Under 35 U.S.C. § 119

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

U.S. Applications			Status (Check One)		
U.S. Application Number	U.S. Filing Date		Patented	Pending	Abandoned
PCT Applications Designating the U.S.					
PCT Application No.	PCT Filing Date	U.S. Serial Number Assigned (if any)			

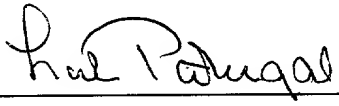
I hereby appoint the following attorney and/or agent to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

David J. Kulik, Reg. No. 36,576.

Please address all correspondence to:

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Potomac, MD 20854

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Applicant: Frank H. PORTUGAL

Serial or Patent No.: _____ Attorney's Docket No.: Cab-001

Filed: filed herewith

For: METHODS FOR IDENTIFYING SPECIES OF SHIGELLA AND E. COLI
USING OPERON SEQUENCE ANALYSIS

**VERIFIED DECLARATION CLAIMING SMALL ENTITY STATUS
UNDER 37 C.F.R. §§ 1.9(f) AND 1.27(b) - INDEPENDENT INVENTOR**

As below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 C.F.R. § 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled METHODS FOR IDENTIFYING SPECIES OF SHIGELLA AND E. COLI
USING OPERON SEQUENCE ANALYSIS described in

- ☒ the specification filed herewith.
☐ Application Serial No.: _____, filed: _____
☐ Patent No.: _____, issued: _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☒ no such person, concern or organization.
☐ persons, concerns, or organizational listed below.*

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27).

FULL NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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